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Leukocytosis-Promoting Factor of *Bordetella pertussis*

III. Its Identity with Protective Antigen

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Leukocytosis-promoting factor (LPF) was purified from the supernatant of a 5-day liquid culture of *Bordetella pertussis* strains Tohama and no. 134. The preparations appeared to be filamentous molecules (2 by 40 nm) and contained histamine-sensitizing and hemagglutinin activities in addition to leukocytosis-promoting activity. Active protection tests in mice demonstrated strong protective activities of 80 international protective units per mg of protein in LPF preparations of each strain toxoided by mild Formalin treatment. Passive protection tests in mice with anti-LPF sera indicated that anti-LPF is one of the protective antibodies.

The protective antigens of *Bordetella pertussis* have been the subjects of intensive studies during the past three decades, but no one has succeeded in characterization of the substance(s) to a satisfactory extent.

Keogh and North (7) reported that the supernatant of liquid culture of *B. pertussis* contained hemagglutinin (HA), which bestowed active immunity on mice against *B. pertussis* infection. Because the immunogenicity was associated with the HA content and the protective potency of the immune serum with the anti-HA potency, they concluded that HA and the protective antigen (PA) were one and the same substance. Pillemer et al. (17, 18) found that PA in the sonic extract of *B. pertussis* is adsorbed quantitatively and selectively onto human red blood cell stroma. They did not assume, however, that HA and PA were one and the same substance. The stroma-protective antigen complex (SPA) prepared by their method has been shown clinically to be effective in protecting children against *B. pertussis* infection (8). Because SPA has not been characterized, there is no way to decide if SPA and HA are the same or different substances. Munoz et al. (13) found both PA and the histamine-sensitizing (HS) factor (HSF) in a fraction of *B. pertussis*.

We described purification of the leukocytosis promoting (LP) factor (LPF) from the extract of agar culture of *B. pertussis* and also the homogeneity of the purified LPF (P-LPF) by electrophoresis, ultracentrifugal analysis, immunoelectrophoresis, and electron microscopy (20). Intravenous injection of mice with P-LPF in an amount of 0.04 μg of protein elicited leukocytosis and histamine sensitization. In addition, LPF, adhering onto the membrane of blood and other cells, manifested HA activity. Adhesion of LPF onto the cell membrane was demonstrated by electron micrography (21). From these findings, we postulated that LPF possesses also the HS, HA, and PA activities.

This report deals with purification of LPF from the supernatant of liquid culture of *B. pertussis* and with the protective activity possessed by LPF.

MATERIALS AND METHODS

Bacterial strains. *B. pertussis* strains Tohama phase I and no. 134 phase I were used. The former strain was the one used previously for LPF preparation (20, 21); the latter strain, the one used for preparation of SPA by Pillemer (17, 18), was supplied by A. C. Wardlow, Department of Microbiology, Glasgow University. Cultures on Bordet-Gengou (BG) medium were freeze-dried for storage.

Culture. The freeze-dried cultures were grown for 72 h and subcultured on BG medium for 24 h at 35 C. The growth was harvested and suspended at a concentration of about 2×10^{10} cells/ml in 200 ml of the liquid medium whose ingredients are shown below. The whole suspension was poured into 10 liters of the liquid medium to give an initial cell concentration of about 4×10^{8} cells/ml.

Liquid culture medium. The liquid culture medium described by Sutherland and Wilkinson (24) and modified by Morse and Bray (9) was used. It contained in grams per 10 liters of distilled water: Casamino Acids (Difco, technical), 100; tris(hydroxymethyl)aminomethane, 60.7; NaCl, 25; soluble starch (Difco), 15; KH_2PO_4, 5; $MgCl_2 \cdot 6H_2O$, 4; DL-

glutamic acid, 2; nicotinamide, 0.3; cysteine-HCl, 0.3; $CaCl_2$, 0.1; $CuSO_4 \cdot 5H_2O$, 0.0075; reduced glutathione, 0.1; and $FeSO_4 \cdot 7H_2O$, 0.1. All ingredients but reduced glutathione and $FeSO_4$ were added to approximately 5 liters of warmed, distilled water. The ingredients were dissolved completely. The solution was adjusted to pH 7.2 with HCl and brought to 10 liters with distilled water. The medium was autoclaved at 121 C for 40 min and cooled immediately. Reduced glutathione and $FeSO_4$ each dissolved in distilled water were sterilized with a membrane filter and added aseptically to the medium to a final concentration of 0.001% (wt/vol). After inoculation (200 ml/10 liters), the medium was stirred thoroughly and dispensed into Roux bottles in 100-ml amounts. The bottles were incubated at 35 C in a flat-laying position to make shallow cultures.

Purification of LPF. The cultures were centrifuged at 15,000 × *g* for 30 min; the clear supernatant fluid was used for purification of LPF. The subsequent purification procedures were the same as those described previously (20).

Cell concentration. The optical density at 650 nm of a cell suspension was compared with that of the National Institute of Health (Japan) opacity standard, and the cell concentration was expressed in international opacity units (IOU).

Protein contents. Protein contents were determined with the copper-Folin phenol reagent and bovine serum albumin (200 μg/ml) as a standard. The reaction mixture was 0.6 ml and read in a semimicro cell.

Titration and neutralization of LP, HS, and HA activities. Determinations of LP, HS, and HA activities and neutralization of LP and HS were performed in the same way as described previously (20, 21).

Titration of agglutinin. Agglutinin was titrated by the method described elsewhere (3). The antigen was the Tohama phase I organisms grown on BG medium for 18 to 20 h and was suspended in phosphate-buffered saline, pH 7.2, containing merthiolate at 0.01% (wt/vol).

HA inhibition test. To each of serial twofold dilutions of kaolin-treated antiserum in 0.1-ml volumes in wells of a plastic tray, 0.1 ml of LPF containing 8 HA units was added. The mixtures were allowed to stand for 30 min at room temperature. Then, 0.2 ml of a 0.5% suspension of chicken erythrocytes was added to each mixture. The highest antiserum dilution causing complete inhibition of hemagglutination was taken as 1 HA inhibition unit.

Antisera. The antisera used were those described previously (21). To absorb anti-LPF from anti-LPF or anti-phase I bacterial serum, a partially purified LPF preparation (SDGC-1) (20, 21) was used at a proportion of 2 mg per ml of a kaolin-treated antiserum. The mixture was allowed to stand at 37 C for 2 h followed by 4 C for 2 days. The flocculus formed was removed by centrifugation at 20,000 × *g* for 30 min. The kaolin treatment of antiserum to remove the nonspecific HA inhibitor will be detailed elsewhere.

Assay for protective activity: (i) active protection test. White female inbred mice of the DDY strain, weighing 14 to 18 g, were used. Each of three consecutive fivefold dilutions of a sample was injected subcutaneously into mice at 0.5-ml doses. After 14 days, each mouse was challenged intracerebrally with 0.025 ml of a cell suspension of strain 18-323 containing about 200 mean lethal dose (LD_{50}) (approximately 50,000 bacilli estimated from the optical density). After observation for 14 days, the mean effective dose (ED_{50}) was calculated by the method of Wilson and Worcester (14, 25); the protective potency was expressed in international protective units (IPU) per mg of protein of LPF.

(ii) Passive protection test. Each of three consecutive fivefold dilutions of a test serum was injected intraperitoneally into mice, weighing 20 to 24 g, in 0.5-ml doses. In 2 h, the mice were challenged intracerebrally with the cell suspension of strain 18-323 described above. The protective potency was expressed in ED_{50} per milliliter calculated from the death rates in 14 days after the challenge.

Alum-precipitated antigen (LPF). Ten milliliters of a Formalin-treated LPF solution (600 μg of protein/ml) was added with 5 ml each of 0.1 M $AlCl_3$ and 0.1 M Na_2HPO_4. The mixture was stirred thoroughly, added with merthiolate to 0.01% (wt/vol), and kept standing overnight at room temperature.

Gel diffusion test. The method of Ouchterlony (15) was employed. Plates were made of 0.8% agarose (Nakarai Chemicals, Ltd., Kyoto) gel in 0.05 M phosphate buffer, pH 8.0, containing 0.5 M NaCl.

Electronmicroscopy. Copper grids, 180 mesh, were coated with carbon film and rendered hydrophilic by glow discharge. A drop of an LPF solution (0.2 mg of protein/ml) was applied on each grid and allowed to dry partially for a few minutes at room temperature. A 1% solution of sodium phosphotungstate, adjusted to pH 7.2 with KOH, was used as a contrasting agent. The specimens were examined at instrumental magnifications of 20,000 to 40,000 times with a Hitachi HU-11B electron microscope.

RESULTS

Growth, pH change, and protein contents of bacterial cells and supernatant in liquid culture of strain Tohama. In Fig. 1, the growth is expressed in optical density of a suspension in phosphate-buffered saline, pH 7.2, of the bacterial cells separated by centrifugation of each sample at 15,000 × *g* for 15 min at 4 C. Each plot is the mean value of five samples.

The bacteria grew exponentially for the initial 3 days and reached the stationary phase in 5 days at a bacterial concentration of about 31 IOU/ml. The time courses of the protein content of the cells and the growth gave similar patterns. The pH of the culture shifted from 7.2 to 8.5 in 5 days; the pH pattern was also similar to that of the growth. The protein content of the supernatant fluid, representing only Folin phenol reagent-reactive small molecular-sized substances, decreased by 18% in 5 days.

Cell-bound and free LP, HS, and HA activ-

ities in liquid culture of strain Tohama. The three activities were determined periodically with samples taken from three Roux bottles (Fig. 2). The cell-bound LP activity increased similarly to the growth, reaching 38 U/ml in 4 days. From the 5th day, when the growth ceased, it began to decrease to 5 U/ml on the

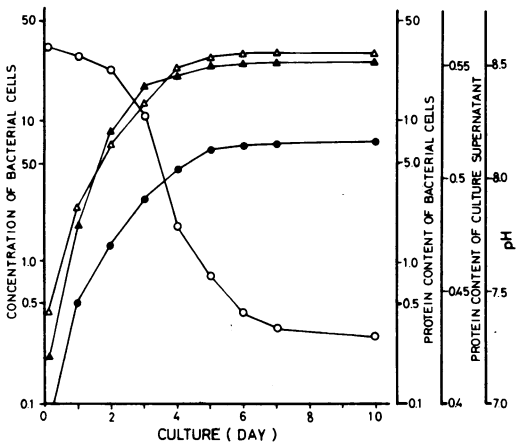

FIG. 1. *Time course of bacterial growth, pH change, and protein contents of bacterial cells and supernatant in liquid culture. Symbols:* Δ——Δ, *concentration of bacterial cells (IOU/ml);* ▲——▲, *pH of whole culture;* ●——●, *protein content of bacterial cells (mg/ml);* O——O, *protein content of culture supernatant (mg/ml).*

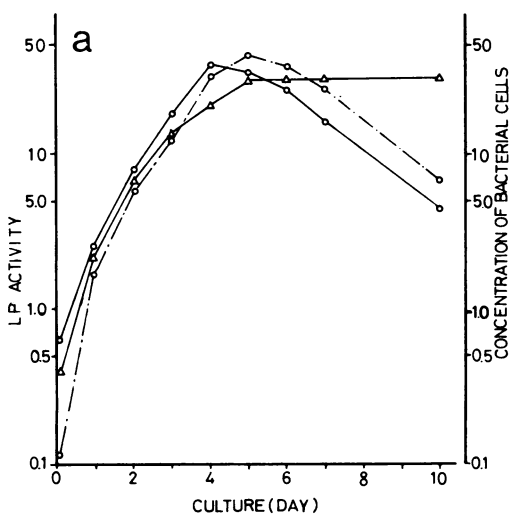

FIG. 2a. *Time course of cell-bound and free LP activities in liquid culture. Symbols:* Δ——Δ, *concentration of bacterial cells (IOU/ml);* O——O, *cell-bound LP activity (U/ml);* O— ·—O, *free LP activity (U/ml).*

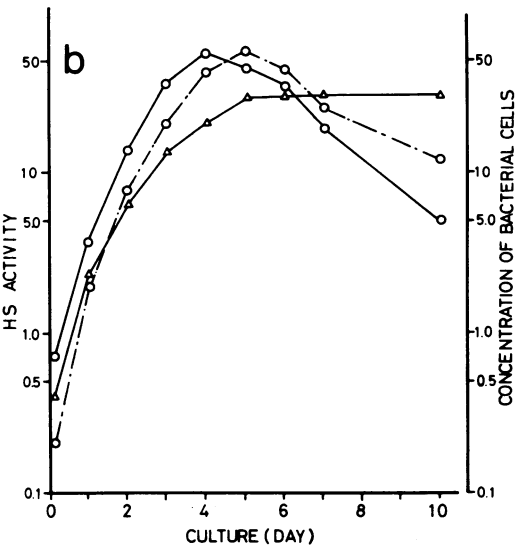

FIG. 2b. *Time course of cell-bound and free HS activities in liquid culture. Symbols:* Δ——Δ, *concentration of bacterial cells (IOU/ml);* O——O, *cell-bound HS activity (HSD_{50}/ml);* O— ·—O, *free HS activity (HSD_{50}/ml).*

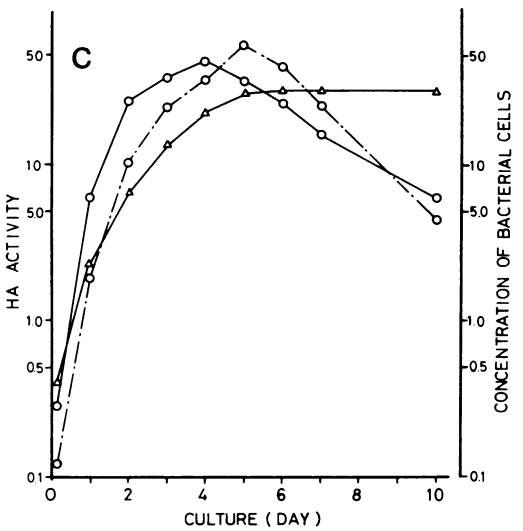

FIG. 2c. *Time course of cell-bound and free HA activities in liquid culture. Symbols:* Δ——Δ, *concentration of bacterial cells (IOU/ml);* O——O, *cell-bound HA activity (U/ml);* O— ·—O, *free HA activity (U/ml).*

10th day. The free LP activity also increased similarly to the growth and the cell-bound LP activity, but it was lower than the cell-bound one during the first 4 days. On the 5th day, however, the free activity was 44 U/ml and the cell-bound activity 33 U/ml. It also decreased

to 7 U/ml on the 10th day or to about 16% of the maximum. In Fig. 2b and 2c, HS and HA activities are shown, respectively. The distribution of these activities in the culture supernatant and the bacterial cells was similar to that of the LP activity.

The foregoing results indicate that the LP-, HS-, and HA-active substance(s) are synthesized in maximum quantities in 5 days in liquid culture and that, on the 5th day, these substances are evenly distributed in the bacterial cells and in the supernatant.

The specific activity of LP in the culture supernatant increased rapidly to 96 U/mg of protein on the 5th day (Fig. 3). That in the cells was kept nearly constant, 5 to 7 U/mg of protein, for the first 4 days and then decreased rather rapidly. A similar tendency was observed with the specific activities of HS and HA (Fig. 1, 2b, and 2c).

Purification of LPF from supernatant of liquid culture. LPF was purified from the supernatant of a 5-day-old culture of strain Tohama and that of no. 134 by consecutive steps of ammonium sulfate fractionation, starch block electrophoresis, and sucrose density gradient centrifugation (20). At each step, the LP, HS, and HA activities of the two strains increased by almost the same rates; however, the HA activity of strain no. 134 was about 10% that of strain Tohama. The LP, HS, and HA activities per mg of protein of P-LPF derived from strain Tohama were 26,500 U, 31,000 HSD_{50}, and 32,500 U, respectively; those from strain no. 134 were 24,000 U, 29,700 HSD_{50}, and 3,000 U, respectively. The recovery of each activity from the culture supernatant was 10 to 15% with either strain.

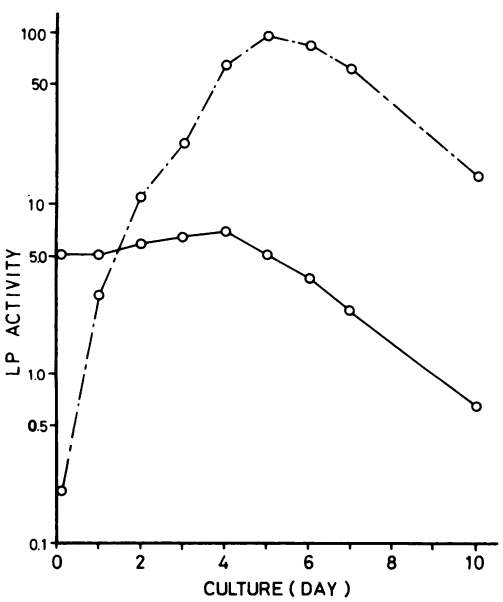

FIG. 3. *Time course of specific activities of cell-bound and free LPF in liquid culture. Symbols:* O——O, *cell-bound LP activity (U/mg of protein);* O—·—O, *free LP activity (U/mg of protein).*

The electronmicrograph of P-LPF derived from culture supernatant of strain no. 134 (Fig. 4) looks similar to that from agar culture of strain Tohama (20), exhibiting filamentous molecules of about 2 by 40 nm in size.

Agar gel diffusion tests with anti-Tohama LPF indicate an immunological difference between LPF derived from strain Tohama and that from no. 134 (Fig. 5). A spur was formed at the conjunction of the precipitin band formed with one antigen and that with the other antigen, indicating that no. 134 LPF and Tohama LPF are not antigenically identical.

Toxoiding LPF by Formalinization. Intraperitoneal or subcutaneous injections with LPF at doses of 15 to 30 μg of protein resulted in deaths of most mice within 14 days due to the "late-appearing toxicity" of LPF; intravenous injections into mice caused body weight decreases followed by deaths usually in 3 to 5 days (21). The minimal lethal dose of P-LPF in a period of 7 days was 4 to 7 μg of protein. It was necessary, therefore, to destroy the lethal toxicity to test LPF for the protective activity. Because it was expected that detoxification of the lethal activity would cause concomitant losses of LP, HS, and HA activities of LPF, Formalinization was attempted with LP, HS, and HA activities as indicators for the lethal toxicity. A 10-ml portion of P-LPF of strain Tohama (600 μg of protein/ml in 0.05 M phosphate buffer, pH 8.0, containing 0.5 M NaCl) was dialyzed against 0.02 M phosphate buffer, pH 7.0, containing 0.5 M NaCl, 0.05 M lysine, and 0.2% Formalin at 25 C with gentle stirring. On the 3rd, 5th, and 7th days, Formalin was added at 0.2% (vol/vol) each time to the outer solution. The LP, HS, and HA activities decreased gradually (Fig. 6); in 4 days after the final addition of Formalin, or in 11 days, all three activities became lower than 0.1% of those before Formalinization. Agar gel diffusion with the Formalin-treated LPF gave a pattern comparable to that with untreated LPF (Fig. 7).

Restoration of toxicity by Formalin-treated LPF. The Formalin-treated LPF regained lethal toxicity upon removal of the Formalin by dialysis at 37 C (Fig. 8). The Formalin-treated LPF (500 μg of protein/ml) of

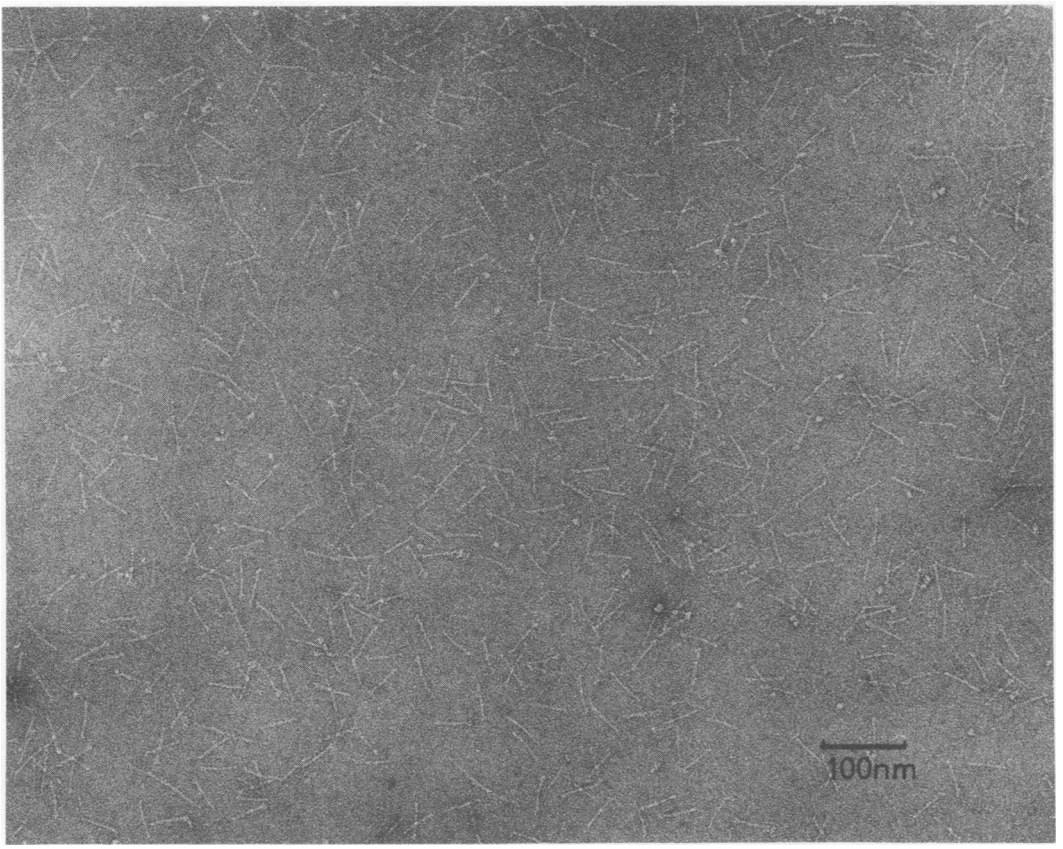

FIG. 4. *Electron micrograph of LPF molecules purified from the supernatant fluid of a liquid culture of B. pertussis phase I, strain no. 134. A 1% solution of phosphotungstic acid in distilled water, adjusted to neutrality, was used for negative staining.*

strain Tohama was dialyzed against 0.02 M phosphate buffer, pH 7.0, containing 0.5 M NaCl, for 3 days at 4 C with daily changes of the buffer and was divided into two portions. One was kept at 4 C and the other at 37 C. Both were subjected periodically to determinations for LP, HS, and HA activities. The activities were kept constant for 21 days at 4 C, whereas they apparently increased at 37 C to about 3.3% of the original activities (Fig. 8).

Active protection tests. Several preparations of LPF, 600 μg of protein/ml, were treated with Formalin as above. Detoxified LPF not freed of Formalin was mixed with $AlPO_4$ gel; the alum-precipitated LPF was diluted in phosphate-buffered saline, pH 7.2, to appropriate concentrations to test for the protective activities in mice. All preparations of LPF elicited high protective activities of about 80 IPU/mg of protein (46.5 to 123 IPU/mg of protein) (Table 1).

Passive protection tests. The antiphase I cell serum bestowed strong protection on mice, with an ED_{50} of 0.011 to 0.014 ml. Anti-LPF sera, viz., antipartially purified LPF, anti-P-LPF, and anti-sheep red blood cell-stroma-adsorbed LPF, also bestowed strong protection with an ED_{50} of 0.01 to 0.03 ml (Table 2). The antiphase I cell and antipurified LPF sera were absorbed with LPF and determined for passive protective potencies (Table 3). The ED_{50} of the antiphase I cell serum did not change appreciably, 0.015 ml before and 0.016 ml after absorption. The corresponding value of the anti-LPF serum was 0.02 ml before absorption, but it markedly increased by absorption, suggesting that the protective antibody in the serum was anti-LPF.

The agglutinin, LP-, HS-, and HA-neutralizing and passive protective titers of various antisera were compared (Table 4). Antiphase I cell serum contained an agglutinin titer higher than 100 times the anti-P-LPF serum, whereas it contained LP-, HS-, and HA-neutralizing

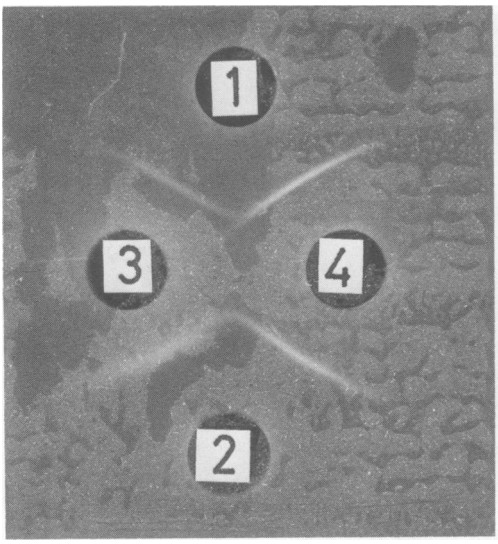

FIG. 5. *Agar gel diffusion tests with LPF obtained from strains Tohama and no. 134. (1) Anti-rabbit Tohama P-LPF serum; (2) anti-rabbit no. 134 P-LPF serum; (3) no. 134 P-LPF (300 μg of protein/ml); (4) Tohama P-LPF (300 μg of protein/ml).*

antibodies about 6% of those in anti-P-LPF. No difference in protective potency was demonstrated between these sera.

DISCUSSION

We purified LPF of *B. pertussis* from the liquid phase of agar culture obtained by freezing and thawing (20, 21). The physicochemically homogeneous P-LPF elicited strong LP and HS activities in the animal and adhered onto the stroma and other cell membranes derived from different animal species (21).

Attempts were made in the present study to purify LPF from liquid culture by simplified procedures. In still culture in a liquid medium, the bacterial population reached the maximum in 4 days. Higher LP activity was found in the bacterial cells than in the cell-free supernatant fluid until the 4th day; after the 5th day, the activity in the supernatant fluid superceded the cell-bound activity. Similar tendencies were observed with HS and HA activities. Periodical protein determinations with cells and supernatant fluid demonstrated no apparent autolysis of the cells. It is likely, therefore, that LPF is released from the cells by some other mechanism than autolysis.

The filamentous molecules possessing LP and HS activities are fimbriae synthesized on the cell surface, which were alluded to by Morse and Morse (9) and will be reported by us more in detail. No explanation can be given for the observation that after the 5th day of incubation, all three activities in the cells and in the supernatant fluid gradually decreased. Production of HA by *B. pertussis* in liquid medium has been reported by Fisher (4, 5), Keogh and North (7), and Sutherland and Wilkinson (24).

From the supernatant fluid of 5-day-old cultures of strains Tohama and no. 134, the latter having been utilized by Pillemer (17) for preparation of SPA, we purified LPF, with a recovery of about 15% by procedures essentially the same as those reported previously (20, 21). The P-LPF of both strains appeared electronmicroscopically to be filamentous molecules of 2 by 40 nm in size, being similar to that obtained from agar culture of strain Tohama (20). All P-LPF preparations contained not only LP activity,

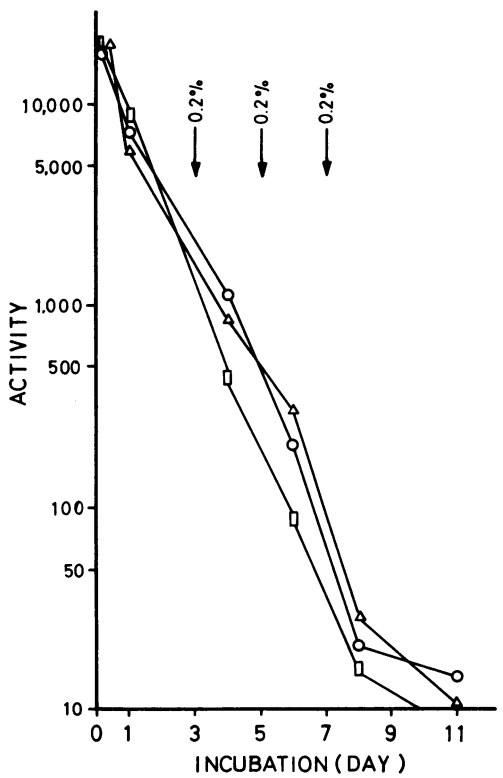

FIG. 6. *Inactivation of LP, HS, and HA activities of P-LPF with Formalin stepwise increasing in concentration. LPF solution was dialyzed against 0.02 M phosphate buffer, pH 7.0, containing NaCl (0.5 M), lysine (0.05 M), and Formalin (0.2%). The Formalin concentration was increased by 0.2% every other day (indicated by arrows) up to 0.8%. Samples were withdrawn at various times to determine the remaining LP, HS, and HA activities. Symbols: Δ——Δ, LP activity (U/ml); O——O, HS activity (HSD_{50}/ml); □——□, HA activity (U/ml).*

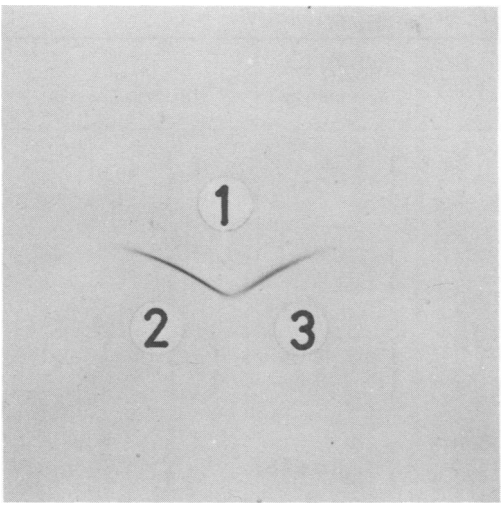

FIG. 7. *Agar gel diffusion tests with Tohama LPF before and after treatment with Formalin. (1) Anti-rabbit P-LPF serum; (2) untreated LPF (600 μg of protein/ml); (3) Formalin-treated LPF (600 μg of protein/ml). A 0.08-ml portion of untreated and Formalin-treated LPF was introduced into the wells. The agar gel slide was incubated at 4 C for 3 days.*

but also strong HS and HA activities. The specific LP and HS activities of LPF derived from the two strains were on the same level; however, the HA activity of LPF of strain no. 134 was about 10% that of strain Tohama LPF. Agar gel diffusion tests revealed that LPF of strain no. 134 is not antigenically identical to Tohama LPF. The antigenic difference might possibly be related to the different HA activities possessed by the two LPF preparations.

From active protection of mice with the Formalin-treated LPF of strains Tohama and no. 134, LPF appeared to be one of PA. The passive protection tests also demonstrated the protective antibody in the anti-LPF serum. It is now clear that the filamentous molecule synthesized on the cell surface of *B. pertussis* phase I and released into the medium during incubation contains four distinct activities, viz., LP, HS, HA, and PA activities. Because the protective antibody in anti-LPF serum was absorbed with LPF, it is apparent that anti-LPF acts as protective antibody. We isolated a 22S antigen with a molecular weight of about 900,000 and possessing a high PA activity from the sonic extract of *B. pertussis* (19, 22). It possessed a PA activity of about 40 IPU/mg of protein, but was completely free from LP, HS, and HA activities. It was innoxious to animals, but LPF was toxic, thus requiring Formalin treatment to test for the antigenicity. Untreated LPF inhibits the antibody production to such antigens as tetanus toxoid and sheep red blood cells (1). Antibody production to LPF itself may also be inhibited. When we isolated the 22S antigen, the PA activity was examined by injecting the mouse with untreated materials. Therefore, the PA activity of LPF may have been overlooked, probably due to the inhibited antibody production. In the present investigation, the protective antibody in the antiphase I cell serum was not absorbed with LPF. Therefore, antiwhole cell serum contains some other antibody than anti-LPF that takes part in protection of the animal against infection. The PA activity possessed by the HA fraction released into the supernatant of liquid culture of this organism was reported by Keogh and North (7), and later by Fisher (5).

Some irreconcilable results were reported by Pillemer (17), who denied the identity of HA and PA and of stroma-adsorbed PA and HA. No convincing results supporting this view were

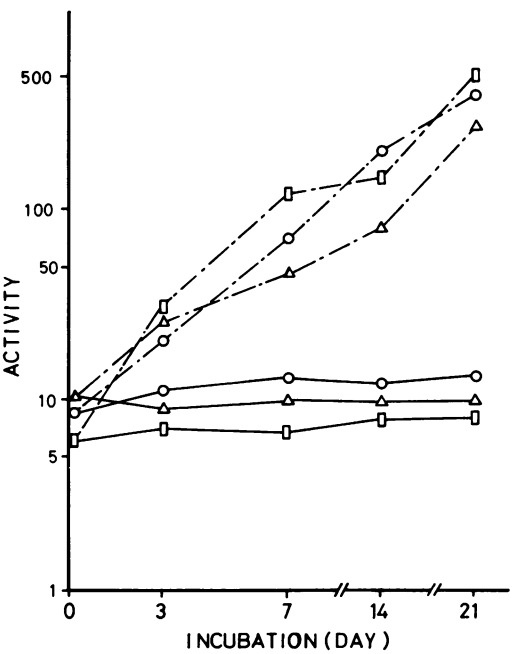

FIG. 8. *Reversion of the LP, HS, and HA activities of Formalin-treated LPF. A Formalin-treated LPF solution was dialyzed against 0.02 M phosphate buffer, pH 7.2, containing 0.5 M NaCl, for 3 days at 1 C. The dialyzed LPF solution was divided into two parts. One was incubated at 4 C and the other at 37 C. Samples were withdrawn at various times to determine the LP, HS, and HA activities. Symbols: △——△, LP activity (U/ml) at 4 C; △—·—△, at 37 C; ○——○, HS activity (HSD_{50}/ml) at 4 C; ○—·—○, at 37 C; □——□, HA activity (U/ml) at 4 C; □—·—□ at 37 C.*

TABLE 1. *Active protection tests of several batches of LPF*

Expt	Immunizing material	Dose inoculated (μg of protein/mouse)	S/16[a]	ED_{50} (1 SD; μg of protein)	Potency (IPU/mg of protein)
I	LPF: Tohama batch A[b]	15 3 0.6	12 5 2	5.4 (3.8–7.8)	49.0
	LPF: Tohama batch B	15 3 0.6	12 6 1	5.3 (3.8–7.3)	50.6
	NIH standard vaccine[c]	1.5 IOU 0.3 0.06	14 7 1	0.37 IOU (0.28–0.49)	0.72 IPU/1.0 IOU
II	LPF: Tohama batch C[b]	15 3 0.6	14 10 2	2.4 (1.8–3.2)	102.0
	LPF: Tohama batch D	15 3 0.6	12 7 4	3.5 (2.2–5.5)	70.6
	NIH standard vaccine	1.5 IOU 0.3 0.06	13 8 2	0.34 IOU (0.24–0.47)	0.72 IPU/1.0 IOU
III	LPF: Tohama batch E	15 3 0.6	13 9 2	3.0 (2.2–4.2)	110.4
	LPF: no. 134 batch A	15 3 0.6	14 8 3	2.7 (1.9–3.7)	123.1
	NIH standard vaccine	1.5 IOU 0.3 0.06	14 5 0	0.46 IOU (0.35–0.60)	0.72 IPU/1.0 IOU
IV	LPF: Tohama batch F	15 3 0.6	12 6 1	5.3 (3.7–7.3)	46.5
	LPF: no. 134 batch B	15 3 0.6	13 8 2	3.4 (2.4–4.7)	72.9
	NIH standard vaccine	1.5 IOU 0.3 0.06	14 8 1	0.34 IOU (0.26–0.45)	0.72 IPU/1.0 IOU

[a] Number of mice surviving per number of mice challenged.

[b] Batches A and C were prepared from extract of solid agar culture as described previously (20). The other batches were prepared from the culture supernatant of the liquid culture.

[c] Japanese standard for the potency determination of pertussis vaccine. The vaccine contains 360 IPU/500 IOU.

given in his report, although his SPA preparation contained HSF activity (18).

We proved that the stroma added to the sonic extract of strain no. 134 cells by the Pillemer method adsorbed quantitatively LP, HS, and HA activities (unpublished data). These findings may indicate that SPA is formed by adsorption of the filamentous molecules in the

TABLE 2. *Passive protection tests with different antisera*

Antiserum[a] to	Serum dilution (0.5 ml/mouse)	S/16[b]	ED_{50} (1 SD; ml)
I			
Control[c]	10	0	
	50	0	
	250	0	
Phase I cells	10	14	0.011 (0.008–0.015)
	50	7	
	250	2	
Phase III cells	10	0	
	50	0	
	250	0	
Partially purified LPF	10	14	0.010 (0.007–0.014)
	50	8	
	250	2	
Purified LPF	10	12	0.016 (0.011–0.021)
	50	6	
	250	2	
SRBC-stromata-adsorbed LPF	10	13	0.019 (0.014–0.025)
	50	5	
	250	0	
II			
Control	3	0	
Phase I cells	10	14	0.014 (0.010–0.018)
	50	6	
	250	1	
Phase III cells	3	0	
Partially purified LPF	10	11	0.024 (0.017–0.033)
	50	5	
	250	0	
Purified LPF	10	14	0.013 (0.009–0.018)
	50	6	
	250	2	
SRBC-stromata-adsorbed LPF	10	10	0.033 (0.020–0.054)
	50	3	
	250	2	

[a] Antisera used in these experiments were the same as described previously (21). SRBC, sheep red blood cells.

[b] Number of mice surviving/number of mice challenged.

[c] Normal rabbit serum.

TABLE 3. *Effect of absorption of LPF antibody from antiphase I cells or anti-LPF serum on passive protection*

Antiserum to	Serum dilution (0.5 ml/mouse)	S/32[a]	ED_{50} (1 SD; ml)
Control[b]	5	0	
Phase I cells	10	26	0.015 (0.011–0.020)
	50	12	
	250	2	
Phase I[c] cells	10	24	0.016 (0.011–0.023)
	50	12	
	250	4	
Purified LPF	10	24	0.020 (0.014–0.028)
	50	10	
	250	2	
Purified[d] LPF	10	7	
	50	1	
	250	0	

[a] Number of mice surviving per number of mice challenged.

[b] Normal rabbit serum.

[c] Antiphase I cells serum absorbed with partially purified LPF.

[d] Antipurified LPF serum absorbed with partially purified LPF.

TABLE 4. *Agglutinin titer, LP, HS, and HA neutralizing titers, and passive protective potencies with different antisera*

Antiserum[a] to	Agglutinin titer	Relative titer of neutralization for		HI titer	Protective potency (ED_{50}/ml)
		LPF	HSF		
Control[b]	<20	<1	<1	<20	<5
Phase I cells	20,480	4	4	800	83
Phase III cells	20	<1	<1	<20	<5
Partially purified LPF	160	16	16	6,400	63
Purified LPF	160	64	64	12,800	71
SRBC-stromata-adsorbed LPF	160	16	16	6,400	41

[a] Antisera used in this experiment was the same as described previously (21). SRBC, sheep red blood cells.

[b] Normal rabbit serum.

cell extract possessing LP, HS, and HA activities onto the stroma, which worked as PA.

Much has been reported on the relation between HSF and PA of this organism and on the protective activity possessed by the HSF-active fraction (6, 11, 13, 16). HSF may be

located in the surface layer of the bacterial cells (12, 23). The most purified HSF preparation obtained by Clausen et al. (2) contained PA as well as LP activities. From the physicochemical properties and the biological activities of HSF reported up until now, the substance isolated by them and the one isolated by us possessing the LP, HS, and HA activities closely resemble each other.

Little has been studied, on the other hand, on the relation between LPF and PA of this organism. Recently Morse and Bray (10) found no protective activity in LPF, which is contradictory to our present finding. The purity of their LPF and their method for antigen preparation could be improved.

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